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#18

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)
)
KLEIN et al.) Group Art Unit: 1641
)
Application No.: 09/155,982) Examiner: V. Portner
)
Filed: October 9, 1998)
)
For: MEANS FOR DETECTING)
BACTERIA OF THE TAYLORELLA)
EQUIGENITALIS SPECIES AND)
THEIR BIOLOGICAL APPLICATIONS)

DEPOSIT DECLARATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Yves DENOUEL, do hereby declare:

[1] That my address is Hôtel du Département - 27 Boulevard de Strasbourg 61017 ALENCON CEDEX;

[2] That I am General Manager of CONSEIL GENERAL DE L'ORNE (FRANCE) employer of Frédéric KLEIN and Dragos GRADINARU;

[3] That a hybridoma obtained using a strain of the species *Taylorella equigenitalis* was deposited at Collection Nationale de Cultures de Microorganismes ; Institut PASTEUR ; 25, rue du Docteur Roux ; F-75724 PARIS CEDEX 15 ; FRANCE as I-2536 on August 3, 2000;

[4] That the deposited material recited in paragraph [3] is a biological material specifically identified in the Klein et al application as originally filed (identification reference : 7C4.10) ; and

[8] That I further declare that all statements made therein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the instant patent application or any patent issuing thereon.

DATE: 17 AVR. 2001 NAME: _____

Yves DENOUEL

Pour le Président du Conseil Général
et par délégation
Le Directeur Général des Services
du Département

Yves DENOUEL

4.2. Results

4.2.1. Bacteriological pathogen detection results

Study period I:

After the intrauterine infection of the pony mare, the pathogen could be reisolated from the uterine exudate on the following day.

T. equigenitalis was cultured in the samples taken in the one to three day interval up to the 31st d.p. inf. from clitoral, cervical and uterine swabs and from uterine exudate and uterine discharge samples.

After the infected pony mare (31 d.p.inf.) had mated with pony stallion 1, the pathogen could be reisolated in the samples taken from the pony stallion from the 4th d.p.inf. The pathogen was detected in all 5 sample materials, with the highest value detected in the pre-seminal fluid. The pathogen could be isolated regularly in the period from the 4th to the 75th d.p.inf. During this period, a significant reduction in the number of small and large T. equigenitalis colonies growing on the nutrient medium was observed. From the 83rd d.p.inf to the 147th d.p.inf., it was only possible to detect isolated bacteria morphologically indicating T. equigenitalis between strongly represented coryneform germs microscopically in Gram preparations (Gram negative soft bacilli or Gram negative cocci). The pure culture required for the identification could not be obtained with the subcultures used. The bacteriological tests conducted on the 161st and 173rd d.p.inf. gave further positive results. In this case, only some small colonies developed from the 7th day of incubation. During the 4th subculture, these colonies supplied further large and small colonies. After the large colony type had been eliminated, the coryneform germs, also growing very slowly on blood agar dishes, caused major difficulties in obtaining pure cultures from the small T. equigenitalis colony type. A summarised overview of the results of the bacteriological tests on pony stallion 1 is given in Table 5.

Table 5: Reisolation of T. equigenitalis in an experimentally infected pony stallion (Animal 1)

d.p.inf.	Body of penis	Urethra[1]	Nav. fossa[2]	PSF[3]	Ejaculum[4]
-34	∅	∅	∅	∅	∅
-14	∅	∅	∅	∅	∅
-1	∅	∅	∅	∅	∅
4	+	+	+	+	/
6	+	+	/	/	/
8	+	∅	∅	+	/
11	+	∅	+	+	+
13	+	+	+	+	/
15	∅	∅	∅	+	/
18	+	∅	+	∅	/
20	+	∅	+	+	/
22	+	+	+	+	/
25	+	+	+	+	/
27	+	∅	+	+	/
75	∅	+	+	+	/
83	∅	∅	∅	∅	∅
91	u.	∅	u.	∅	/
110	∅	∅	∅	u.	/
131	∅	u.	u.	∅	/
138	u.	u.	u.	u.	/
147	∅	∅	u.	∅	/
161	∅	/	+	∅	/
173	∅	∅	∅	+	+

Key: ∅ Negative pathogen detection

\+ Positive pathogen detection

u Microscopic detection of uncertain cultures

/ No samples taken

[1] HR = Harnröhre
[2] EG = Eichelgrube
[3] VS = Vorsekret
[4] Eja = Ejakulat

Study period II:

The successful initiation of the infection in pony stallion 2 was verified with positive results in the bacteriological study of the samples taken from the 3rd d.p.inf. In this case, a constant decrease in the T. equigenitalis colonies growing on the nutrient media was observed up to the 21st d.p.inf. It was clearly noted that, from the 17th d.p.inf., no more large colony variants developed. From the 3rd d.p.inf. to the 14th d.p.inf., both small and large colonies were observed, with the latter visible from 48 hours of incubation time and the former only visible between the 6th and the 8th day of incubation. From the 17th d.p.inf., only small colonies, only visible from the 6th to the 8th day of incubation, developed. In the third subculture of these small colonies, further large and small colony variants were recorded. From the 24th d.p.inf. to the 84th d.p.inf. and from the 131st to the 240th d.p.inf., all the tests to reisolate T. equigenitalis were negative. On the 260th and 289th d.p.inf. and on the 367th and 386th d.p.inf., isolated Gram negative soft bacilli or coccal bacteria could be detected between strongly represented coryneform germs in the bacteriological examination of navicular fossa swabs in Gram preparations, which were composed of morphologically uncertain colonies from the original smear. However, since the subcultures used to obtain the pure cultures required for the final identification of T. equigenitalis were not successful, it was only possible to express an uncertain result. From the 333rd d.p.inf. to the 355th d.p.inf. and on the 398th d.p.inf., complete pathogen detection was again obtained, i.e. the pathogen was identified morphologically in the Gram preparation and biochemically and serologically after subculture. In this case, only small colonies, visible from the 8th day of incubation could be observed. During the subculture of these colonies, large colonies developed, which were isolated in the third subculture on the 5th day of incubation and proliferated in the fourth subculture on the 3rd day of incubation.

The strongly represented coryneform germs in the samples represented a significant handicap in the bacteriological test for T. equigenitalis in the infection test phases, in which no more large colony types developed in the original smear, due to their massive development and their identical growth rate and similar colony morphology in relation to the small T. equigenitalis colony type.

Since Test animal 2 was not yet sexually mature at the beginning of the study (first ejaculum was free of sperm), it was only possible to take a few pre-seminal fluid or ejaculum samples due to insufficient or low sexual libido up to the 159th d.p.inf.

An overview of the results of the bacteriological T. equigenitalis detection tests on pony stallion 2 is given in Table 6.

Table 6: Reisolation of T. equigenitalis in an experimentally infected pony stallion (Animal 2)

d.p.inf.	Body of penis	Urethra	Nav. fossa	PSF	Ejaculum
-16	Ø	Ø	Ø	/	/
-9	Ø	Ø	Ø	/	/
-2	Ø	Ø	Ø	Ø	Ø
3	Ø	Ø	+	+	/
7	Ø	Ø	+	+	/
10	Ø	Ø	+	/	/
14	Ø	Ø	+	/	/
17	Ø	Ø	+	/	/
21	Ø	Ø	+	/	/
24	Ø	Ø	Ø	/	/
31	Ø	Ø	Ø	/	/
35	Ø	Ø	Ø	/	/
42	Ø	Ø	Ø	/	/
49	Ø	Ø	Ø	/	/
56	Ø	Ø	Ø	/	/
84	Ø	Ø	Ø	/	/
91	Ø	Ø	+	/	/
98	Ø	Ø	+	/	/
131	Ø	Ø	Ø	/	/
146	Ø	Ø	Ø	/	/
159	Ø	Ø	Ø	Ø	Ø
173	Ø	Ø	Ø	Ø	Ø
219	Ø	Ø	Ø	Ø	Ø
240	Ø	Ø	Ø	Ø	Ø
260	Ø	Ø	u.	Ø	Ø
289	Ø	Ø	u.	Ø	Ø
319	Ø	Ø	Ø	Ø	Ø
333	Ø	Ø	Ø	+	Ø

Table 6 (continued): Reisolation of T. equigenitalis in an experimentally infected pony stallion (Animal 2)

d.p.inf.	Body of penis	Urethra	Nav. fossa	PSF	Ejaculum
351	∅	∅	∅	+	∅
355	∅	+	∅	∅	∅
367	∅	∅	u.	u.	u.
386	∅	∅	u.	∅	∅
398	∅	∅	∅	u.	+

Key: ∅ Negative pathogen detection
+ Positive pathogen detection
u Microscopic detection of uncertain cultures
/ No samples taken

The bacteriological study for T. equigenitalis on the uterine, cervical and clitoral swabs taken from the pony mare four times in the interval from 14 to 30 days before mating with pony stallion 2. On the third and fifth day after mating with the pony stallion mentioned above, T. equigenitalis could be detected in culture on cervical and uterine swabs. In this case, isolated large colonies could be observed on the third day of incubation and further abundant small colonies on the seventh day of incubation. On the 14th day after the first mating, Taylorella were isolated from the clitoral swab for the last time. The further four bacteriological tests on clitoral, cervical and uterine swabs for each 10-day interval were negative.

4.2.2. Clinical progression of Taylorella infection

In the course of the infection study, both infected pony stallions showed no clinical symptoms which could be attributed to the effect of T. equigenitalis. Symptoms of slight colic were observed in pony stallion 2 on the 237th and 238th d.p.inf.

4.2.3. Serological study results

The results of the serological study for both pony stallions are summarised in Tables 7 and 8.

Table 7: Serological study results for pony stallion 1

d.p.inf.	SAT	HT	PHA	CFT	IFT
-40	1:5	1:5	∅	∅	∅
0	∅	∅	∅	∅	1:10
7	∅	1:5	∅	∅	1:10
14	∅	1:5	∅	∅	∅
22	∅	1:5	∅	∅	∅
28	∅	1:5	∅	∅	∅
75	∅	1:5	∅	∅	∅
83	∅	1:5	∅	∅	∅
91	∅	∅	∅	∅	∅
110	∅	∅	∅	∅	∅
138	∅	1:5	∅	∅	∅
168	∅	∅	∅	∅	∅

Table 8: Serological study results for pony stallion 2

d.p.inf.	SAT	HT	PHA	CFT	IFT
-1	1:5	Ø	Ø	Ø	Ø
3	1:5	Ø	Ø	Ø	Ø
7	1:10	Ø	Ø	Ø	Ø
10	1:10	Ø	Ø	Ø	Ø
14	1:10	Ø	Ø	Ø	Ø
17	1:5	Ø	Ø	Ø	Ø
21	1:10	Ø	Ø	Ø	Ø
24	1:5	Ø	Ø	Ø	Ø
28	Ø	Ø	Ø	Ø	Ø
35	1:5	Ø	Ø	Ø	Ø
42	1:5	Ø	Ø	Ø	Ø
49	1:5	Ø	Ø	Ø	Ø
56	1:5	Ø	Ø	Ø	Ø
84	1:5	Ø	Ø	Ø	Ø
91	1:5	Ø	Ø	Ø	Ø
98	1:5	Ø	Ø	Ø	Ø
131	1:5	Ø	Ø	Ø	Ø
146	1:5	Ø	Ø	Ø	Ø
156	1:5	Ø	Ø	Ø	Ø
219	1:5	Ø	Ø	Ø	Ø
240	1:5	Ø	Ø	Ø	Ø
260	1:5	Ø	Ø	Ø	Ø
388	1:10	Ø	Ø	Ø	1:10
403	1:5	Ø	Ø	Ø	Ø
423	1:10	Ø	Ø	Ø	1:10

4.2.4. Haematological study results

The results of the study of the red and white blood cell count for pony stallion 1 and pony stallion 2 are given in Tables 9, 10, 10 and 12.

Table 9: Haematological study results for a pony stallion infected with T. equigenitalis (Animal 1)

d.p.inf.	Hb, old	Hb, new	HC	Ery	MCV	MCH	MCHC
-34	11.1	6.89	30	7.29	41.2	15.2	37.0
0	11.5	7.14	34	8.83	38.5	13.0	33.8
7	13.3	8.25	36	9.41	38.3	14.1	36.9
14	13.6	8.44	36	7.53	20.5	18.1	37.8
28	15.1	9.37	39	11.14	35.0	13.5	38.7
83	11.8	7.32	32	9.55	33.5	12.4	36.9
91	10.2	6.38	28	8.25	33.9	12.4	36.4
169	12.8	7.84	35	7.84	45.8	16.8	36.6
173	14.1	8.75	39	8.09	48.2	17.4	36.2

Table 10: Differential blood count for a pony stallion infected with T. equigenitalis (Animal 1)

d.p.inf.	Bas	Eos	Myel	Granulocytes				sm.Ly	l.Ly	Mon	Leu
				Imm	nbf	bf	sgm				
-34	0	5	0	0	0	9	34	34	19	0	6450
0	0	6	0	0	13	12	31	31	6	2	5800
7	0	5	0	1	17	10	31	31	3	2	4000
14	0	3	0	0	1	16	22	52	4	0	7300
28	0	3	0	0	0	11	32	47	7	0	6400
83	0	4	0	0	0	12	22	57	3	1	6300
91	0	1	0	0	0	28	11	53	6	1	6050
169	0	3	0	0	0	2	30	43	20	2	6650
173	0	2	0	0	2	3	22	37	34	0	7600

Table 11: Haematological study results for a pony stallion infected with T. equigenitalis (Animal 2)

d.p.inf.	Hb, old	Hb, new	HC	Ery	MCV	MCH	MCHC
-1	10.3	6.39	32	10.19	43.4	10.1	32.2
3	10.9	6.74	37	7.59	48.7	14.4	29.5
7	9.4	5.83	36	8.08	44.6	11.6	26.1
10	15.8	9.80	41	10.49	39.1	15.1	38.5
14	13.8	8.56	35	8.01	43.7	17.2	39.4
17	13.7	8.50	36	8.55	43.1	16.0	38.1
21	15.3	9.49	39	9.49	42.1	16.5	39.2
24	14.0	8.69	38	9.04	42.0	15.5	36.8
28	13.9	8.63	33	7.91	41.7	17.6	42.1
35	13.7	8.50	37	8.72	42.4	15.7	37.0
42	13.3	8.25	34	8.81	38.6	15.1	39.1
49	11.6	7.20	35	8.12	43.1	14.3	33.1
56	14.0	8.69	38	9.50	40.0	14.7	36.8
84	11.9	7.38	33	7.84	42.1	15.2	36.1
91	10.7	6.64	38	7.23	52.6	14.8	28.2
98	10.5	6.52	37	8.99	41.2	11.3	28.4
131	12.8	7.94	34	9.21	36.9	13.9	37.6
146	13.2	8.19	35	9.01	38.8	14.7	37.7
156	12.9	8.01	36	8.64	41.7	14.9	35.8
219	12.6	7.82	34	7.64	44.5	16.5	37.1
240	11.2	6.95	31	7.10	43.7	15.8	36.1
260	14.4	8.94	38	8.78	43.3	16.4	37.9
388	15.8	9.80	38	8.50	44.7	18.6	41.6
403	12.3	7.63	34	8.11	41.9	15.2	36.2
423	13.8	8.56	38	9.36	40.6	14.7	36.3

Table 12: Differential blood count for a pony stallion infected with T. equigenitalis (Animal 2)

d.p.inf.	Bas	Eos	Myel	Granulocytes				sm.Ly	l.Ly	Mon	Leu
				Imm	nbf	bf	sgm				
-1	0	0	0	0	1	10	34	34	17	4	7950
3	0	2	0	0	0	2	20	27	47	2	8700
7	0	1	0	0	0	3	20	25	48	3	12000
10	0	2	0	0	1	3	27	43	22	2	10100
14	0	1	0	0	0	2	31	26	38	2	11500
17	0	1	0	0	0	5	23	31	36	4	10850
21	0	4	0	0	0	3	31	21	38	3	11150
24	0	0	0	0	0	6	21	25	48	0	9550
42	0	3	0	0	0	10	27	38	20	2	10200
49	0	1	0	0	0	11	29	13	45	1	8850
56	0	0	0	0	0	2	26	38	34	0	12600
84	0	3	0	0	2	10	23	11	50	1	9750
91	1	6	0	0	0	2	33	13	43	2	9550
98	0	2	0	0	0	7	31	35	22	3	10450
131	0	6	0	0	0	5	27	30	31	1	9200
146	2	4	0	0	0	6	22	25	41	0	8350
156	0	3	0	0	1	8	27	35	25	1	8350
219	0	1	0	0	0	7	32	23	36	1	8650
240	0	0	0	0	5	6	3	30	56	0	4450
260	0	1	0	0	0	6	24	22	47	0	10300
388	0	1	0	0	1	7	39	29	23	0	9750
403	0	6	0	0	1	6	20	23	40	4	7050
423	0	6	0	0	0	8	27	28	30	1	10400

4.2.5. Post mortem T. equigenitalis detection in culture

In the post mortem study of the urinary and genital organs of both pony stallions, T. equigenitalis could only be reliably detected in the external genital organs. In the studies of the ancillary genital glands, gonads and bladders, on the other hand, microscopically uncertain bacteria, i.e. bacteria indicating T. equigenitalis in the Gram preparation, were observed. However, these bacteria could not be identified further during to an absence of growth in the subculture study, so an uncertain result could only be expressed. The T. equigenitalis colonisation of the positive organs in the bacteriological study was very insignificant except for the primary and secondary navicular fossae.

The study of the urinary tract organs (expect for the bladder) and the lymph nodes were negative.

The horse blood agar dishes used in the study of the organs of pony stallion 1 supplemented with different quantities of oxacillin and lincomycin did not show any advantage over nutrient media without antibiotic supplements. In the event of positive pathogen detection, growth of small T. equigenitalis colonies was only observed from the ninth day of incubation. In the subsequent subcultures, germ growth developed between the second and fifth day of incubation. In the post mortem study of the organs removed from pony stallion 2, in the case of positive results, incubation periods of 7 days (navicular fossae) to 9 days (urethra, glans, body of penis) were required to observe developed small colony variants. In the subculture, these variants also showed growth between the second and fifth day of incubation.
The results of the post mortem bacteriological studies are summarised in Table 13.

Table 13: Post mortem bacteriological detection of T. equigenitalis in two experimentally infected pony stallions

Study material	Stallion 1	Stallion 2
Lnn. inguinales supff.	∅	∅
Lnn. renales	∅	∅
Lnn. sacrales intt.	∅	∅
Lnn. sacrales extt.	∅	∅
Lnn. coeliaci	∅	∅
Lnn. ilici medd.	∅	∅
Lnn. hypogastrici	∅	∅
Lnn. lumbales aortici	∅	∅

Table 13 (continued): Post mortem bacteriological detection of T. equigenitalis in two experimentally infected pony stallions

Study material	Stallion 1	Stallion 2
Kidney	∅	∅
Pelvis of kidney	∅	∅
Ureter	∅	∅
Bladder	u.	u.
Testicles	u.	u.
Epididymal head	u.	u.
Epididymal body	∅	∅
Epididymal tail	u.	u.
Vas deferens	∅	∅
Ampullae of vas deferens	u.	∅
Prostate gland	∅	u.
Seminal vesicles	∅	u.
Bulbourethral glands	∅	∅
Urethra (proximal section)	+	∅
Urethra (median section)	+	+
Urethra (distal section)	+	+
Glans	S	S
Primary navicular fossa	+	+
Secondary navicular fossa	+	+
Prepuce	S	S
Body of penis	+	S

Key: ∅ Negative pathogen detection

\+ Positive pathogen detection

u Microscopic detection of uncertain cultures

S Dishes overgrown with spore formation

4.2.6. Pathological study results

In the anatomicopathological studies of the internal and external genital organs and urinary tract organs conducted after the euthanasia of both pony stallions and in the histopathological studies of the above-mentioned organs of pony stallion 1, no modifications could be observed.

In the histopathological study of the urinary and genital organs of pony stallion 2, the following pathological modifications were observed. The seminal vesicle comprised isolated circumscribed lymphohystiocytic infiltrations (Fig. 1).

In the testicles, isolated giant cells developed and sperm production was considerably reduced (Fig. 2). In conjunction, the epididymal ducts proved to be partially free of sperm (Fig. 3).

In the region of the distal urethra, lymph follicles were observed. The study of the secondary navicular fossa detected subepithelial lymphohistiocytic infiltrates.

Fig. 1: Lymphohistiocytic infiltrates in the seminal vesicle (19-fold magnification)

Fig. 2: Giant cell in lumen of epididymal ducts (125-fold magnification)

Fig. 3: Epididymis, duct partially free of sperm (19-fold magnification)

4.2.7. Immunohistological detection of T. equigenitalis

In the IFT study of the organ and tissue sections of pony stallion 2 using the double labelling technique, isolated fluorescences developed at the same location in the tissue when stimulated with UV light and green light in the seminal duct of the testicles (Fig. 4, Fig. 5) and in the lumen of the seminal vesicle.

Specific fluorescences were also observed in the mucous membrane folds of the glans and urethra. In the study of the primary and secondary navicular fossae, amplified fluorescent bacteria could be observed, particularly in the latter. With the stimulation of the FITC conjugate, both bacilli and cocci showed a marked membrane fluorescence, while with rhodamine conjugate stimulation, only bacilli were fluorescent.

The immunohistological studies of the regional lymph nodes, kidney, ureter, bladder, epididymis, vas deferens, ampulla of the vas deferens, prostate gland, bulbourethral glands and prepuce were negative.

Fig. 4: Fluorescence in the epididymal duct with FITC stimulation (immersion in oil, 1000-fold magnification).

Fig. 5: Fluorescence in the epididymal duct with rhodamine stimulation (same section of image as Fig. 4, immersion in oil, 1000-fold magnification).

4.2.8. Immune serum production in rabbits

All three vaccinated rabbits showed a rapid rise in the antibody titre, which reached a plateau after the booster dose (Fig. 6).

Fig. 6: Progression of the anti-T. equigenitalis antibody titre measured in PHA in the serum of 3 immunised rabbits

Serum antibodies

Animal 1
Animal 2
Animal 3

d.p.vacc.

The pooled rabbit serum had the following anti-T. equigenitalis antibody titres with the serological detection methods applied: RAT 1:128, PHA 1:1280, SAT: 1:320, HT: 1:160, CFT: 1:512, IFT: 1:512. In the RAT, agglutination only occurred with Actinobacillus equuli when undiluted serum was used, which, however, did not occur at the next highest dilution factor of 1:2. No cross-reactions with any other bacterial strains tested could be observed.

4.2.9. Monoclonal antibody characterisation

The Balb/c mice immunised with 3 different antigen preparations showed anti-T. equigenitalis antibodies with the ELISA technique 2 weeks after the primary immunisation. The titres increased further after the booster injection, reached their peak between the 90th and 130th d.p.vacc. and began to fall after that. The table below gives the titre dynamics.

Table 14: Titre progression in immunised Balb/c mice

d.p.vacc.	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6
0	negative	negative	negative	negative	negative	negative
14	1:2560	1:2560	1:640	1:640	1:320	1:640
21	1:10240	1:10240	1:5120	1:2560	1:2560	1:2560
35	1:5120	1:5120	1:5120	1:5120	1:5120	1:5120
70	1:10240	1:5120	1:10240	1:10240	1:10240	1:10240
90	1:10240	1:5120	1:10240	1:10240	1:20480	1:20480
110	1:10240	1:5120	1:10240	1:5120	1:10240	1:10240
130	1:5120	1:5120	1:10240	1:5120	1:5120	1:5120
142	1:5120	1:5120	1:5120	1:5120	1:5120	1:5120
207					1:5120	1:5120
Final titre	1:5120	1:5120	1:5120	1:5120	1:2560	1:2560

In the fusion reactions, in which the murine myeloma cell line P3X63-Ag8 was used as the fusion partner, no stable antibody-producing hybridoma cell lines could be established. On the other hand, in the 3 fusion reactions with the heteromyeloma cell line CB-F7 as the fusion partner, 89 antibody-producing hybridoma cell lines were established. After the study of 45 different clones with the Western blot technique, the clones TF I 10D5, TF II 8D4, TF III 10G5, TF III 11B5, TF III 11B5, TF III 11E5, TF III 7D4, TF III 7D4, TF III 3G3, TF III 3E8 were selected from the total quantity for further characterisation due to their different reactions with the Immunoblot technique.

Figure 7 documents the Immunoblot reactivity of the mAb directed against T. equigenitalis. The mAb TF II8D4 was the only of the mAb selected not to show a clear reaction with a specific protein. The total NC band (No. 9) was diffusely stained. The accompanying negative control (No. 10) and the conjugate control (No. 11) showed a weak non-specific reaction with a 19 KD protein. This reaction was present on all the NC bands.

Fig. 7: mAB Immunoblot reactivity against T. equigenitalis

Key: No.:

1 Molecular weight marker
2 TF I 10D2
3 TF III 7D4
4 TF III 10G5
5 TF III 3E8
6 TF III $11^{E}5$
7 TF III 11B5
8 TF III 3G3
9 TF II 8D4
10 Kufu 2
11 Conjugate control
12 Mouse serum

The immunoglobulin subclasses, the ELISA and IFA titre of the residual hybridoma and the relative molecular weights of the T. equigenitalis proteins detected with the Immunoblot technique are summarised in Table 15.

Table 15: Properties of anti-T. equigenitalis mAb

mAb	Immunoglobulin subclass	ELISA / IFT titre	Molecular weight of detected proteins
TF I 10D2	IgG_2a	1:128/1:10	13 kD
TF II 8D4	IgM	1:8192/1:320	no reaction
TF III 11E5	IgG_2a	1:8192/1:320	56 kD
TF III 11B5	IgG_2a	1:8192/1:320	70 kD, 117 kD
TF III 10G5	IgG_1	1:8192/1:10	35 kD
TF III 7D4	IgG_2a	1:5121/1:5	22 kD
TF III 3G3	IgG_2a	1:128/1:10	47 kD, 70 kD
TF III 3E8	IgG_1	1:128/1:10	47 kD

The study of the selected mAb with the ELISA technique with respect to their ability to detect different T. equigenitalis isolates showed differences in the antigen structure of this species. The T. equigenitalis strain I/3 is not detected by the mAb TF 11E5 (Fig. 12) and the strain BW 26 is not detected by the mAb TF I 10D5 with the ELISA technique (Fig. 9). In addition to these qualitative differences, quantitative differences are also observed. These marked differences in the extinctions measured occurred, with the exception of the mAb TF II 8D4, in all the other mAb tested. The results of the specificity test are represented in Figures 9 to 19 in the table appendix.

In the study of potential cross-reactions of all the mAb used with representatives of different bacterial species, no antigenic affinities could be detected. Potential antigenic affinity of T. equigenitalis with Streptococcus zooepidemicus and Streptococcus equi cannot be proven due to the non-specific fixation of the conjugate used represented in the figure (Fig. 19).

The results of the examination of the mAb with respect to their ability to detect different Taylorella isolates and representatives of different bacterial species with the ELISA technique are illustrated in Figures 9 to 16.

While the mAb Kufu 2 used as the negative control did not react with the Taylorella strains under test or with the other bacterial species, the mouse immune serum used as the positive

control showed weak-positive reactions (extinction > 300) with the majority of the bacterial species used. The strongest reaction occurred with Haemophilus somnus, Micrococcus albus and Bordetella bronchiseptica (Fig. 17).

With the IFT technique, of all 8 mAb under study, only the mAb of the clones TF II 8D4, TF II 11B5 and TF III 11E5 showed very good fluorescence up to a dilution factor of 1:320 (Fig. 8). The remaining mAb only reacted at dilution factors from 1:5 to 1:10. The mAb detected the baccillary form and coccoid form of T. equigenitalis present in the culture material in a similar fashion.

Fig. 8: mAb TF III 11E5 reaction with the IFT technique

5. Discussion of results

Bacteriological and clinical study results

After the mating of pony stallion 1 with the T. equigenitalis-positive pony mare, the pathogen was isolated in swabs from the body of the penis, urethra, navicular fossa, pre-seminal fluid and ejaculum. After the mating of pony stallion 2 with the pony mare, T. equigenitalis was also successfully transmitted to the pony mare, thus demonstrating the reciprocal transmissibility of the pathogen by means of mating.
TAINTURIER et al. (1982a) also succeeded in transmitting T. equigenitalis by means of mating from one infected mare to a total of 8 stallions.

In a transmission study with trotter stallions and mares, SCHLÜTER et al. (1991) were able to demonstrate that mating with an infected mare did not necessarily result in pathogen transmission. The authors did not succeed in detecting the pathogen in 2 stallions, which had mated with a T. equigenitalis-positive mare several times.
To determine to what extent the adhesion of the infection after the transmission of the pathogen is breed- or age-dependent or whether individual predisposing factors are involved, further studies are required.

The CEM pathogen was most frequently detected in pony stallion 1 in the pre-seminal fluid with 11 positive results, while the swabs from the urethra only gave 6 positive results. With 10 positive pathogen detections, respectively, the results of the bacteriological studies of the body of the penis and navicular fossa equal those for the pre-seminal fluid. In the ejaculum, the pathogen was detected twice in a total of three samples taken.
A completely different picture was observed with respect to the bacteriological studies in study period II in the bacteriological test on pony stallion 2. In spite of the intraurethral inoculation with T. equigenitalis, the pathogen was only detected on the 355th d.p.inf. in the urethra. The ejaculum studies gave one positive result and that of the pre-seminal fluid gave 4 positive results. With a total of 8 Taylorella detections, the navicular fossa swabs from this animal showed the most frequent detection of the pathogen, whereas, in the swabs from the body of the penis, the CEM bacterium was not detected over the entire study period of 433 days.

The negative pathogen results in the swabs from the body of the penis could be related to the differing mode of infection in study period I, since in the natural mating, the entire body of the penis of pony stallion 1 came into contact with the Taylorella-infected vaginal mucous membrane, while pony stallion 2 was infected by the intraurethral route.
TAINTURIER et al. (1982a) were also unable to isolate the pathogen in the body of the penis in a total of 3 pony stallions infected by the intraurethral route.

Comparing the positive study results in both pony stallions shows that the pathogen cannot be cultured in a reproducible manner from any part of the external stallion genitals, with respect to a high accuracy of detection of the direct pathogen detection, although T. equigenitalis was more frequently reisolated from swabs from the navicular fossa overall in both study periods. Only the study of all the samples involved in the pathogen detection (swabs from the body of the penis, navicular fossa, urethra, pre-seminal fluid and ejaculum) offers the best condition for successful detection of T. equigenitalis.
PLATT et al. (1978) described a procedure similar to study period II for bacteriological reisolation in 4 artificially infected pony stallions. The authors obtained the highest rate of T. equigenitalis detection in swabs from the navicular fossae, whereas the bacteriological studies of the samples from the body of the penis were mostly negative.
LORIN et al. (1984) succeeded in detecting the pathogen eleven times in bacteriological studies on samples materials from 11 naturally infected stallions in swabs from the navicular fossa, while only 4 samples from the urethra and one sample from the body of the penis and ejaculum, respectively, were positive.

The results with respect to the high rates of T. equigenitalis detection in navicular fossa swabs obtained in some reisolation studies and illustrated in the literature supported the hypothesis formulated by SIMPSON and EATON-EVANS (1978) and HAZARD et al. (1979), that, as the main site of colonisation, the navicular fossa in the stallion is the anatomical equivalent to the clitoral sinus in the mare.
With its low acidic and moist medium, the secondary navicular fossa, constantly filled with smegma, offers the ideal colonisation site for T. equigenitalis.

The persistence of T. equigenitalis lasting up to the 173rd d.p.inf. in pony stallion 1 to the 389th d.p.inf. in pony stallion 2 underlines the possibility of the pathogen persisting for several months already demonstrated by TIMONEY and POWELL (1982) and TIMONEY and STRICKLAND (1982).
The fact that, after the infection of the stallion with the CEM pathogen, rapid germ elimination may occur, is shown by an infection study by TAINTURIER et al. (1982a), which only succeeded in obtaining positive pathogen detections on the 9th d.p.inf. in a pony stallion infected by the intraurethral route. Further pathogen detections were negative.

The positive results obtained in pony stallion 1 in the bacteriological study on the pre-seminal fluid on the 15th d.p.inf. and on the pre-seminal fluid and ejaculum on the 173rd d.p.inf., along with the positive pathogen detections on the 333rd and 351st d.p.inf. in the pre-seminal fluid and on the 398th d.p.inf. in the ejaculum of pony stallion 2, with, at the same time, negative or uncertain results of the swabs from the body of the penis, navicular fossa and urethra studied support the assumption expressed by SCHLÜTER et al. (1990) on the possible colonisation of the internal genital organs in the stallion by T. equigenitalis. The authors formulated this suspicion due to pathogen detections in the pre-seminal fluid and sperm with concurrently negative results in swabs from the external genitals in a naturally infected stallion after a total of 8 treatment cycles.

The reduction observed in the T. equigenitalis colonies grown in the bacteriological study of the samples taken from both stallions and the transformation of the colony type from large, rapid-growth to the small, slow-growth colony type must be seen to be related to the inhibitory effect on the physiological bacterial flora described by SWERCZEK (1979c), DOLAN et al. (1984) and VAISSAIRE (1986) and the asymptomatic progression of CEM infection in the stallion.
Since no data exists in the literature on the transformation of T. equigenitalis colony growth in stallion cultures, it is only possible to refer to publications on infection studies in mares.
KANEMARU et al. (1988) infected thoroughbred and pony mares with culture material composed of the large colony type or of the small colony type. In this way, the mares infected with the large colony type showed typical CEM symptoms (endometritis, cervicitis, vaginitis), while the mares infected with the small colony showed no reactions at all.

SAHU and WEBER (1982) inoculated three pony mares with small colony type culture material and described a dependency of the severity of the clinical symptoms on the speed of the transformation from the small to the large colony type. While, in the first pony mare, almost exclusively large colonies were observed on the 3rd d.p.inf., the transformation in the two other pony mares lasted up to the 14th d.p.inf. Accordingly, strongly distinctive CEM symptoms were only observed in the first pony mare.

On the basis of the growth of the small colony type in the bacteriological study of pony stallion 2 on the 351st d.p.inf., it can be assumed that, during the mating of the pony stallion with the pony mare, exclusively slow-growth Taylorella were transmitted to the genital tract of the mare. Although, as early as the 3rd d.p.inf., large Taylorella colonies could be cultured in the uterine and cervical swabs, the pony mare only showed a slightly increased exudation. This clinical progression of the infection in the mare, which is not consistent with the above-mentioned results obtained by SAHU and WEBER (1982), can be explained by the reinfection of the pony mare in the mating. In the studies by TIMONEY et al. (1979c), SAHU et al. (1980) and FRIEDRICH (1989), reinfected pony mares showed no or only very mild clinical symptoms after Taylorella infection.

Since the small T. equigenitalis colony type which developed in the course of the bacteriological studies was difficult to distinguish morphologically from other very slow-growth accompanying germs, particularly coryneform germs, and, as a result, the subculture required for the diagnosis of T. equigenitalis to obtain pure Taylorella cultures was considerably difficult or partly impossible to make, the mAb produced within the scope of this study open up new possibilities in the identification of uncertain culture materials.

KANEMARU et al. (1988) and KAMADA et al. (1987) also describe problems in the identification of the small CEM pathogen colony type due to the similar slow growth rate of various accompanying germs.

It is only possible to make assumptions on the cause of the positive pathogen detection on the 333rd d.p.inf. after the phase with negative or uncertain study results lasting from the 131st d.p.inf. to the 319th d.p.inf. The pathogen content required for the bacteriological detection of T. equigenitalis could result from the repeated elimination of semen from the pony stallion due to sexual activity. Further studies are required to determine to what extent modifications in hormonal status or modifications in the physiological genital flora are involved in this process.

The growth period of the Taylorella colonies between 2 (large colony types) and 9 days (small colony type) indicate the possible time required for the detection of T. equigenitalis in swabs from stallions. The incubation time required in the primary isolation studies by WARD et al. (1984) in 39% of isolates also lasted more than 6 days.

In both study periods, no clinical symptoms which could be connected with T. equigenitalis infection developed in either pony stallion.
These observations are in line, except for a publication by LORIN et al. (1984), with the asymptomatic progression of CEM in stallions described in the international literature (PLATT et al. 1978, TAYLOR et al. 1978, TAINTURIER et al. 1979, 1981a, 1982a, ECKSTEIN 1983, ODA et al. 1983, VAISSAIRE et al. 1986, 1987, Köhler 1987).
LORIN et al. (1984) observed, in an infected stallion, one-sided, high-grade painful, indurated and therapeutically responsive orchitis, with concurrent detection of mycoplasmas in the ejaculum. However, the question in relation to which of the pathogens mentioned above this condition can be attributed remains open.

Haematological and serological study results

In the haematological monitoring of both pony stallions, over the entire study period, the results of the red and white blood cell counts were, with a few exceptions, within the limits of the specifications defined by KOLLAKOWSKI and KELLER (1990) and KIEFERNDORF and KELLER (1990). In pony stallion 1, on the 7th d.p.inf, leucopaenia with left shift developed, which may be the result of a confrontation of the body's cell defence with an antigen. The leucopaenia with left shift detected in the second pony stallion on the 240th d.p.inf. is not so much seen to be related to the T. equigenitalis infection as to the minor colic developing on the 237th and 238th d.p.inf.

The results of the haematological results underline the nature of CEM as a local infection of the genital mucous membranes and are consistent with the unimpaired clinical condition in both pony stallions throughout the study period, except for the minor colic condition in pony stallion 2.

The serological reactions with the SAT, HT and IFT techniques in both stallions under study should be considered as non-specific reactions.

The reactions with the IFT technique in both stallions up to a serum dilution factor of 1:10 should, according to the studies by TAINTURIER et al. (1981b), be assessed as negative.

The above-mentioned authors detected fluorescent antibodies in horses (mares and stallions) without any contact with T. equigenitalis up to a serum dilution factor of 1:10 and, after comparing these sera with sera from infected mares, established the titre limit for IFT at a titre factor of 1:20.

The fact that, as early as the day of infection, pony stallion 1 had reactive antibodies at a serum dilution factor of 1:10 with the IFT technique, indicates the accuracy of this limit. The fluorescent antibodies observed by TAINTURIER et al. (1981a) in the serum of two artificially infected pony stallions at 1:5 and 1:10 serum dilution factors were also considered as negative reactions.

The agglutination reactions observed with the SAT and HT techniques in the serum dilution factors should also be considered as negative in view of the international literature.

After serological studies, BENSON et al. (1978) and DAWSON et al. (1978) considered agglutination reactions with the SAT technique as positive from a serum dilution factor of 1:80. MACMILLAN and KIDD (1986) found similar agglutination reactions in horse sera, without the animals having been in known contact with CEM. 86% of the sera tested reacted, 19% of which at serum dilutions of 1:40 and over. These positive reactions could not be increased with the Coombs test and proved to be sensitive to 2-mercapto-ethanol. For these reasons, the positive agglutination reactions were attributed to non-specific IgM fixation.

Although positive antibody detection were obtained with the CFT technique by PITRE et al. (1979) and with the PHA technique by SCHLÜTER et al. (1990) in bacteriological positive stallions, no serum antibodies could be detected in the infection study conducted on both pony stallions, also using these serological detection methods.

Post mortem study results

In the post mortem bacteriological and immunohistological studies, T. equigenitalis could not be detected in the regional lymph nodes in culture or using immunohistological methods.
Since studies of this kind had not been previously conducted on stallions, it is only possible to refer to the results obtained by ACLAND et al. (1983), which detected the CEM bacterium in the lymph nodes belonging to the genital tract of the mare using fluorescent serological means and thus deduced the basis for the serological reaction of the mare.

The negative bacteriological studies of the kidney, pelvis of the kidney, ureter, epididymal body, vas deferens and bulbourethral glands could be substantiated by negative immunohistological results.

In the cultures developed from the bladder, testicles, epididymal heads, epididymal tails of both pony stallions and in the ampullae of the vas deferens of pony stallion 1 and the prostate gland and seminal vesicle of pony stallion 2, it was possible to detect isolated, soft Gram-negative bacillary or coccoid bacteria morphologically indicating T. equigenitalis after the production of Gram preparations of uncertain colony material. Since the further culture of these bacteria was not successful and, in immunohistological studies, positive results were only observed in the study of the testicles and seminal vesicle in pony stallion 2, it is only possible to make assumptions on the identity of the bacteria detected microscopically in the above-mentioned organs.
In addition to the possibility that the bacteria observed actually consisted of Taylorella, which had colonised in the organs with uncertain bacteriological results at a quantitatively very low level, in view of the literature, other bacterial species should be included in differential diagnosis considerations.
According to MACKINTOSH (1981), other microaerophilic Gram negative soft bacilli or coccoid bacteria, such as the haemophilus, neisseria, branhamella and bordetella species, should be taken into consideration in differential diagnosis. However, the above-mentioned types were previously only very rarely isolated from mares. In spite of its Gram negative staining action, the species found in genital tract mucous membranes of stallions, Moraxella urethralis, is well distinguished from T. equigenitalis due to the chain or diploid arrangement observed microscopically.

The positive post mortem pathogen detections in the entire urethra, the primary and secondary navicular fossae, glans and body of the penis show that, in the stallion, the external genitals are the first to be colonised. On the basis of the observations made in the pre- and post mortem bacteriological studies on the level of Taylorella colonisation, the primary and secondary navicular fossae are confirmed as the main colonisation site. The pathogen colonisation of the urethra, the external mucous membrane of the glans and the body of the penis takes place at a low quantitative level.

The microscopic detection of soft Gram negative bacilli and coccoid bacteria in the post mortem bacteriological study of the testicles and the isolated occurrence of specific fluorescence indicating fixation of rabbit immune serum and mAb TF III 11E5 antibodies on antigen-bearing structures in the lumen of the epididymal duct with the IFT technique imply, however, a low quantitative colonisation of the CEM pathogen in the testicles of pony stallion 2.

Since morphological modifications were observed by LAPAN (1990) after the in vitro effect of extracts of 56 T. equigenitalis strains on Vero and Y1 cells, cytotoxins formed by T. equigenitalis should be considered as the cause of the degenerative modifications on the germ epithelium.

The fluorescence observed in the immunohistological pathogen detection in the seminal vesicle at the same location when stimulated with green or ultraviolet light, which result from the fixation of antibodies of the rabbit immune serum and the mAb used, could be considered to confirm the suspected T. equigenitalis colonisation in the bacteriological study of this ancillary genital gland. The quantitative level of pathogen colonisation should be estimated as being very low, as in the testicles.

However, the analysis of the literature gives several indications of a possible colonisation of the ancillary genital glands by T. equigenitalis.

In studies on the virulence factors of the CEM pathogen by LAPAN (1990), it was possible to detect the ability of D-Mannose-resistent adhesion in 47.3% of the 56 T. equigenitalis strains under study. The adhesion ability detected, which is present in a large number of pathogenic bacteria colonising the mucous membrane, should be considered in this context as the basic condition for colonisation of the genital mucous membranes by T. equigenitalis.

A further indication is the anti-T. equigenitalis IgA activity detected by PARR (1990) with the ELISA technique in 98.8% of the pre-seminal fluid samples studied. This may result from the colonisation of the mucous membranes of the genital tract by T. equigenitalis.

The literature only contains results from post mortem bacteriological studies by PLATT et al. (1978) on a pony stallion sacrificed on the 22nd d.p.inf. The authors isolated the CEM bacterium in the distal urethra, the primary navicular fossa and the prepuce and concluded from their results, that ascendant Taylorella infection had not taken place.

In the anatomicopathological study of the organs removed from both pony stallions, no pathological modifications were observed on the external and internal sections of the genital tract or on the parts of the urinary tract. In the histopathological study of the organs of the above-mentioned organ systems in pony stallion 1 also, no indications of inflammatory modifications were observed.
These results are consistent with those of PLATT et al. (1978) on a pony stallion infected with T. equigenitalis and sacrificed on the 22nd d.p.inf. The authors only described mild non-specific testicular degeneration.

Unlike the results of the histopathological studies on pony stallion 1, in similar studies on the second pony stallion, divergent results were obtained in the testicles, epididymises, seminal vesicles, in the region of the urethra and the secondary navicular fossa. The isolated giant cells detected histopathologically in pony stallion 2 in the testicles should be viewed in relation to the decreased sperm production observed.
According to SEFFNER (1986) and SCHULZ (1991), giant cells develop in the event of sperm production disorders under the effect of various harmful substances as a result of meiosis disorders with the formation of hyperploid giant cells. Bacteria could represent one of these etiological factors.
Therefore, the partially sperm-free epididymal ducts should be considered as the consequence of lower sperm production.
The histopathological modifications detected in the second pony stallion in the seminal vesicles, subethelial in the region of the urethra and the secondary navicular fossae in the form of local lymphohistiocytic infiltrates in the interstitium of the glands indicate that the local immune system has encountered an antigen.

However, since T. equigenitalis could only be detected immunohistologically in the seminal vesicle and in the testicles, the probability that the CEM bacterium is responsible for the modifications in both these organs is slim.

Immune serum production in rabbits

With the antigen used for immunisation in conjunction with the adjuvant applied, it was possible to induce a high serum antibody titre in all three immunised rabbits.
In the SAT specificity test, the undiluted pooled serum only agglutinated with Actinobacillus equuli. In spite of this, it was noted in the immunohistological study of the navicular fossae with the double labelling technique, that under FITC stimulation, both bacillary bacteria and cocci reacted, while under rhodamine stimulation, only bacilli produced fluorescence. This observation implies a non-specific reaction of the immune serum with the IFT technique with coccal bacteria. Since these bacteria were not detected by the mAb used but the mAb reacted with the use of culture material both with bacillary and coccoid forms of T. equigenitalis, it can be concluded that the cocci detected by the rabbit immune serum are coccoid forms of T. equigenitalis.
Various results are contained in the literature on cross-reactions of immune sera produced in rabbits against T. equigenitalis, which are discussed in relation to the specificity of the mAb.

Anti-T. equigenitalis mAb characterisation results

The mAb directed against T. equigenitalis (except for TF II 8D4) detect, with the Western blot technique after separation in SDS-PAGE under non-reducing conditions, proteins with a relative molecular weight of 13, 22, 35, 47, 56, 70 and 117 kD of the Taylorella strain used for immunisation. 2 proteins in the region of 70 and 117 kD and 47 and 70 kD were detected by the mAb TF III 11B5 and TF III 3G3, respectively.
The results contained in the literature on immunogenic external membrane proteins are essentially based on studies using polyclonal rabbit immune sera and sera from infected horses.

LAPAN (1990) detected different immunogenic proteins in the molecular weight region of 14 to 130 kD in all of the 56 T. equigenitalis strains studied with the Western blot technique, with a polyclonal rabbit serum in SDS-PAGE under reducing conditions. However, since the author does not give any molecular weights of the individual proteins detected by the immune serum, it is only possible to compare with the individual results using estimations. Accordingly, 14, 22, 39, 43, 45, 48, 53, 57 and 130 kD proteins were detected by the rabbit serum.
SUGIMOTO et al. (1988) isolated the external membrane of T. equigenitalis and detected the main proteins in SDS-PAGE under non-reducing conditions with a molecular weight of 15, 27, 33, 41, 48 and 50 kD. The authors were able to detect using the Western blot technique a specific reaction of the 41 kD protein for T. equigenitalis infection, by comparing the reaction of mare sera, which were sampled before and after the T. equigenitalis infection.

Since native antigen material was used with the ELISA technique, the antibody-producing hybridoma cells were selected with respect to the ability of the murine antibodies to detect immunogenic T. equigenitalis surface structures. Using the mAb TF III 11E5 and TF I 10D5, it was succeeded in detecting differences of the antigen structure of different T. equigenitalis isolates for the first time. The mAb TF III 11E5 did not react with the Taylorella strain I/3 belonging to the DNA Fingerprint group I according to LAPAN (1990) with the ELISA technique, while all the other strains under study were only detected with insignificant quantitative differences in the extinction measured by the antibody. In addition, the Taylorella strain BW/26 was also not detected with the ELISA technique by the mAb TF I 10D5. The mAb TF I 10D5 detected the remaining T. equigenitalis isolates under test, but significant differences in the extinctions measured were recorded.
The other 6 mAb selected bonded with all the Taylorella strains under test, but some very marked differences were observed in the extinctions measured. Since equal quantities of antigen and antibody were used in the test system applied, these differences indicate both quantitative and qualitative differences in the composition of the epitopes detected by the mAb. The quantitative difference between the T. equigenitalis isolates could lie in a quantitatively different expression of certain epitopes, while qualitative differences would be attributable to a different affinity of the mAb for the epitopes of the individual T. equigenitalis strains under study detected.

In the literature available, no data is given on similar previous studies with mAb.
In comparative studies by LAPAN (1990), 56 T. equigenitalis strains were compared using SDS-PAGE with respect to their protein pattern. None of the strains studied showed qualitative or quantitative differences in the protein pattern.
In previous immunological studies on the antigen composition of the CEM pathogen, only polyclonal rabbit immune sera, which did not enable the detection of any immunological differences between the Taylorella strains under test with the SAT and co-agglutination techniques, were used (TAYLOR et al. 1978, KITZROW et al. 1979, DABERNAT et al. 1980b, MAZUROWA 1985, KAMADA et al. 1987, SELBITZ et al. 1987).
With the IFT technique, TER LAAK and WAGENAARS (1990) observed no serological differences in any of the 20 T. equigenitalis strains under study using a goat immune serum.
CORBEL and BREWER (1982) detected 11 precipitating antigens with protein characteristics with polyclonal rabbit immune serum on ultrasound and phenol extracts of 5 T. equigenitalis strains with the immunoelectrophoresis technique, with no detectable differences between the isolates used.
LAPAN (1990) was also unsuccessful in detecting differences in the antigenic structure of 56 isolates with an immune serum produced in rabbits with the Immunoblot technique.
On the basis of the ELISA reaction of the mAb with the representatives of the genome groups detected according to LAPAN (1990), no relationship between the genome group and mAb reaction has been previously demonstrated. The question in relation to the extent to which the different reactions of the selected mAb with the T. equigenitalis isolates under test could be attributed to the division of T. equigenitalis strains in different antigen groups remains open. In order to test the possibility of the use of the mAb as an epidemiological marker, it is necessary to test their reaction with a larger number of T. equigenitalis strains.

The non-existent reaction of the mAb TF II 8D4 with the Immunoblot technique may have various causes, which must be determined in further studies. One of the possible causes would be that this mAb detects a discontinuous epitope, i.e. an epitope on the cell membrane surface, which loses its antigenic properties after the denaturing treatment of the bacterial cells with SDS due to the destruction of the tertiary structure. A further possibility would be that this antibody is directed against lipopolysaccharides (LPS) which are strongly represented in the cell walls of Gram negative bacteria.
Studies by CORBEL and BREWER (1982) and SIGOMOTO et al. (1988) demonstrated that LPS are involved in the immune reaction produced by T. equigenitalis. CORBEL and

BREWER (1982) detected an LPS protein antigen and a polysaccharide antigen with a rabbit serum. SUGIMOTO et al. (1988) detected an immunogenic LPS located in the external membrane and also an immunogenic non-LPS-component with the cross-immunoelectrophoresis technique with sera from infected mares, corresponding, in the opinion of the authors, to the components detected by CORBEL and BREWER (1982).

In the clarification of possible cross-reactions of the 8 different mAb selected with representatives of various bacterial species, no specific immunological reactions occurred with the ELISA technique, whereby it is impossible to make a statement on the antigenic properties of T. equigenitalis with Streptococcus equi or Streptococcus zooepidemicus due to the non-specific fixation of the POD conjugate used by the latter bacterial species.

In the literature, various authors repeatedly report on cross-reactions of immune sera produced against T. equigenitalis with a wide variety of bacteria, or it is concluded, due to the immunological reactions of human and bovine sera with T. equigenitalis, that the CEM pathogen and other bacteria present in the human or bovine genital tract have common antigenic properties.

TAYLOR et al. (1978) detected cross-reactions with Brucella abortus, Pasteurella multocida, Haemophilus influenzae, Yersinia pseudotuberculosis and Yersinia enterocolitica with a rabbit immune serum with the tube agglutination technique at a serum dilution factor of 1:10 to 1:20. The serum also reacted with the RAT technique undiluted with Moraxella osloensis (NCTC 10465) and Actinobacillus species (NCTC 10803) or up to a 1:2 dilution with Haemophilus influenzaemurium (NCTC 11135), Neisseria elongata subspecies glycolytica (NCTC 11050) and Pasteurella pneumotropica (NCTC 10609). NEILL et al. (1984) described agglutinations of an undiluted rabbit immune serum with Actinobacillus species (NCTC 10803) and Acinetobacter lwoffi, a bacterium, which, according to VAISSAIRE et al. (1987) belongs to the normal saprophytic flora of the stallion.

After the adsorption of antibodies from a rabbit hyperimmune serum by the protein A positive staphylococcus strain Cowan I, KITZROW et al. (1979) observed a weak reaction with Haemophilus suis with the co-agglutination technique.
On the other hand, DABERNAT et al. (1980b), TAINTURIER ET AL. (1981c), MAZUROWA (1985) and SELBITZ et al. (1987) did not detect any cross-reactions of the immune sera they produced in rabbits with other bacterial species.
The mAb reaction with the IFT technique showed the ability of all mAb to react with T. equigenitalis at different intensities with this detection method. The mAb TF II 8D4 and TF III 11B5 have emerged as being particularly suitable for use in IFT.

The establishment of hybridoma cells, producing mAb directed against Taylorella equigenitalis, opens up new paths in research on the epidemiology of this equine genital disease. Since the mAb TF II 8D4 and TF III 11B5 detect all the Taylorella strains under test with the ELISA technique, did not show any cross-reactions with previously tested bacterial species and these antibodies are able to react with the IFT technique, it is possible to establish a rapid test based on IFT to study smears of genital swabs and genital secretions of stallions and mares for T. equigenitalis. In addition, these mAb could be used with the IFT technique to identify uncertain culture material which, as the infection study conducted also demonstrated, results in difficult diagnosis in the event of asymptomatic T. equigenitalis carriers. For this, the identification of the antigenic determinants of the CEM bacterium detected by the mAb TF II 8D4 would be required.
TER LAAK et al. (1989) and TER LAAK and WAAGENAARS (1990) already used a hyperimmune serum developed in goats to identify T. equigenitalis cultures, which self-agglutinated under specific culture conditions. This methods to identify uncertain bacteria could be more specific with the use of mAb.

In order to obtain a more accurate profile of the specificity of mAb produced within the scope of this study, further comparative studies with bacterial species that have not been tested to date, which were described as cross-reactive in the literature, should be conducted. In this context, the study of possible common antigenic properties of T. equigenitalis with bacteria of the physiological genital flora of stallions and mares according to their bacteriological culture would be of interest.

Furthermore, studies on common antigenic properties of T. equigenitalis with pathogenic and opportunistic bacterial species colonising the male genital tract, which are considered as the cause of the reaction of T. equigenitalis with human serum, would be possible.

6. Conclusions

In infection studies with Shetland ponies, it was possible to demonstrate the sexual transmissibility of Taylorella equigenitalis, wherein the CEM bacterium was transmitted from a pony mare to a pony stallion and from a pony stallion to a pony mare.

Since, in the bacteriological studies of genital swabs, it was not possible to determine any site in the external stallion genitals, from which the pathogen can be reisolated in a reproducible manner, in order to obtain a high accuracy of detection in the stallion, swabs from the body of the penis, the primary and secondary navicular fossae, the urethra, pre-seminal fluid and ejaculum should be studied.

However, since, in all the bacteriological studies conducted in 2 pony stallions, T. equigenitalis was most frequently isolated from the navicular fossa swabs and in the post mortem bacteriological studies of the urinary and genital organs of both pony stallions and in the immunohistological study of organ sections from pony stallion 2, the highest Taylorella concentration was found in the primary and secondary navicular fossae of the stallions, the primary and secondary navicular fossae can be considered as the main Taylorella equigenitalis colonisation site in the male genital tract, equivalent to the clitoral sinus in the mare.

The sudden positive pathogen detections in pony stallion 2 after the intensive semen elimination training give grounds for the assumption that infected mating stallions, which gave negative results in stud hygiene studies before the beginning of the mating season, may suddenly transmit the pathogen massively as a result of sexual activity in the mating season.

The time required for the primary isolation of T. equigenitalis which varies between 2 and 9 days according to colony size in this infection study, which was associated with a further few days for subculture and to ensure the diagnosis, results in a clear pathogen detection time.
Since in the stallion, it is first of all necessary to account for the development of the slow-growth small colony type in asymptomatic T. equigenitalis carriers, blood agar dishes should be incubated for at least 10 days to detect Taylorella in suspect stallions to avoid false negative results.

Using the immunohistological study of organ and tissue sections from pony stallion 2, it was possible to confirm the suspicion expressed in the post mortem bacteriological study with respect to the T. equigenitalis colonisation in the testicles and semen vesicles. Since this antigen detection method confirmed the assumption expressed repeatedly in the literature on the possibility of T. equigenitalis colonisation in the internal genital organs of the stallion for the first time, the question as to whether this colonisation of the CEM pathogen generally took place in the stallion needs to be answered.

Since no specific antibodies could be detected using the SAT, HT, CFT, PHA and IFT techniques in either of the infected pony stallions during the course of the infection study, serological studies on T. equigenitalis antibodies should be considered unsuitable to detect Taylorella infection in stallions.

The results of the haematological studies in both infected pony stallions underline the local and asymptomatic nature of T. equigenitalis infection in the stallion.
This statement should also be taken into consideration in relation to the unclear results of the anatomicopathological studies conducted post mortem in both horses.

The lymphohistiocytic infiltrates that could be detected interstitially in the seminal vesicle, and subepithelially in the urethra and secondary navicular fossa in the histopathological study of the genitals and the decreased sperm production observed in the testicles could be considered as a result of the effect of Taylorella equigenitalis or the cytotoxins produced by this species. With the sperm production disorder observed, fertility disorders in stallions as a result of Taylorella infection should be studied further.

However, since the histopathological studies of the genital organs of both pony stallions did not give a uniform picture, further studies are required with respect to the effect of the CEM pathogen on the genital organs of stallions.

The fact that the mAb TF II 8D4 and TF III 11B5 detect all Taylorella isolates tested to date with the ELISA technique and also react with the IFT technique, the use of these two mAb in a rapid IFT test to study smears of genital swabs and genital secretions and suspect colony material for Taylorella equigenitalis would appear to be reasonable.

The possibility to use the mAb as an epidemiological marker to divide T. equigenitalis isolates into antigen groups on the basis of the different reaction patterns with the mAb should be verified by testing the reaction of the selected mAb with a larger number of T. equigenitalis strains.

Using the mAb, it was not possible to detect any common antigenic determinants of 12 different T. equigenitalis isolates and 21 other bacterial species with the ELISA technique. This result indicates the specificity of the mAb directed against T. equigenitalis. However, before the recommended mAb are used with the IFT technique to detect T. equigenitalis, further studies on cross-reactions with bacteria in the physiological genital flora of stallions and mares should be conducted.

7. Summary

The purpose of this work was to study the progression and effects of T. equigenitalis infection and the colonisation sides of the CEM bacterium in the stallion.

To resolve this question, intra vitam bacteriological, clinical, serological and haematological studies were conducted on two artificially infected Shetland pony stallions. A post mortem anatomicopathological and histopathological study of the urinary and genital organs and the regional lymph nodes was conducted with respect to modifications induced by T. equigenitalis, along with pathogen detection in culture. The organs removed from one stallion also underwent immunohistological pathogen detection using a hyperimmune serum produced in rabbits and monoclonal antibodies directed against T. equigenitalis. The hyperimmune serum and the monoclonal antibodies were also tested with respect to their specificity using 12 T. equigenitalis isolates and 23 other bacterial species.

In the infection study, no clinical symptoms related to T. equigenitalis occurred. In the serological studies, no T. equigenitalis-specific antibodies were detected in the SAT, HT, PHA, CFT and IFT. Similarly, the results of the haematological studies did not allow any conclusions as to the confrontation of the infected pony's body's cell defence system with the CEM bacterium. In the bacteriological studies, the primary and secondary navicular fossae were established as the main T. equigenitalis colonisation site in the stallion.

In the immunohistological study, T. equigenitalis was detected in the seminal vesicles and testicles of a pony stallion with the IFT double labelling technique.

With monoclonal antibodies directed against T. equigenitalis, it was possible to detect, for the first time, differences in the antigenic structure of representatives of the T. equigenitalis species and to open up new paths in research on the epidemiology of this equine genital disease. The monoclonal antibodies make it possible to speed up CEM diagnosis and increase the level of accuracy of detection.